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In re Application of:

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Jorgen Nedergaard LARSEN

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For: NOVEL RECOMBINANT ALLERGENS

CLAIM FOR PRIORITY

Hon. Commissioner of
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Sir:

Applicant hereby claims priority under 35 U.S.C. Section 119 based on Danish Patent Application No. 0364/98 filed March 16, 1998.

A certified copy of the priority document is submitted herewith.

Respectfully submitted,

Dated: June 3, 1999

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Patent application No.: 0364/98

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This is to certify the correctness of the following information:

The attached photocopy is a true copy of the following document:

- The specification, claims and drawings as filed with the application on the filing date indicated above

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TAASTRUP 08 APR 1999

Karin Schlichting
Head Clerk

Novel Recombinant Allergens

FIELD OF THE INVENTION

The present invention relates to novel recombinant allergens which are non-naturally occurring mutants derived from naturally occurring allergens. Further, the invention relates to a method of preparing such recombinant allergens as well as to pharmaceutical compositions, including vaccines, comprising the recombinant allergens.

BACKGROUND OF THE INVENTION

Genetically predisposed individuals become sensitized (allergic) to antigens originating from a variety of environmental sources, to the allergens of which the individuals are exposed. The allergic reaction occurs when a previously sensitized individual is re-exposed to the same or a homologous allergen. Allergic responses range from hay fever, rhinoconductivitis, rhinitis and asthma to systemic anaphylaxis and death in response to e.g. bee or hornet sting or insect bite. The reaction is immediate and can be caused by a variety of atopic allergens such as compounds originating from grasses, trees, weeds, insects, food, drugs, chemicals and perfumes.

However, the responses do not occur when an individual is exposed to an allergen for the first time. The initial adaptive response takes time and does usually not cause any symptoms. But when antibodies and possibly T cells capable of reacting with the allergen have been produced, any subsequent exposure may provoke symptoms. Thus, allergic responses demonstrate that the immune response itself can cause significant pathological states which may be life-threatening.

The antibodies involved in atopic allergy belong primarily to immunoglobulins of the IgE class. IgE binds to mast cells and basophils. Following complex formation of a specific allergen with IgE bound to mast cells, IgE is cross-linked on the cell surface, thus resulting in the physiological response of the IgE-antigen interaction. Degranulation results in the release of i.a. histamine, heparin, a chemotactic factor for eosinophilic leukocytes, leukotrienes and C4, D4 and E4, which cause prolonged constriction of the bronchial smooth muscle cells. The resulting effects may be systemic or local in nature.

The antibody-mediated hypersensitivity reactions can be divided into four classes, namely type I, type II, type III and type IV. Type I allergic reactions is the classic immediate hypersensitivity reaction occurring within seconds or minutes following antigen exposure. The symptoms are mainly due to specific IgE production and reaction.

Commonly, allergic reactions are observed as a response to protein allergens present e.g. in pollens, house dust mites, animal hair and dandruff, venoms, and food products.

In order to eliminate the strong allergic reactions, carefully controlled and repeated exposure to the allergen (by parenteral or oral administration in increasing doses over a fairly long period of time) is commonly used in order to desensitize the patient. The exact mechanism is not known, but is believed to involve production of non-IgE antibodies such as IgG and IgA which are specific for the allergen in question.

In WO 97/30150, a population of protein molecules is claimed, which protein molecules have a distribution of specific mutations in the amino acid sequence as compared

to a parent protein. From the description, it appears that the invention is concerned with producing analogues which are modified as compared to the parent protein, but which are taken up, digested and presented to T cells in the same manner as the parent protein (naturally occurring autoallergens). Thereby, a modified T cell response is obtained. Libraries of modified proteins are preparied using a technique denoted PM (Parsimonious Mutagenesis).

In WO 92/02621, recombinant DNA molecules are described, which molecules comprise a DNA coding for a polypeptide having at least one epitope of an allergen of trees of the order Fagales, the allergen being selected from *Aln g* 1, *Cor a* 1 and *Bet v* 1. The recombinant molecules described herein do all have an amino acid sequence or part of an amino acid sequence that corresponds to the sequence of a naturally occurring allergen.

WO 90/11292 relates i.a. to isolated allergenic peptides of ragweed pollen and to modified ragweed pollen peptides. The peptides disclosed therein have an amino acid sequence which corresponds either to the sequence of the naturally occurring allergen or to naturally occurring isoforms thereof.

Antibody-binding epitopes

X-ray crystallographic analyses of F_{ab}-antigen complexes has increased the understanding of antibody-binding epitopes. According to this type of analysis antibody-binding epitopes can be defined as a section of the surface of the antigen comprising atoms from 15 - 25 amino acid residues, which are within a distance from the atoms of the antibody enabling direct interaction. The affinity of the antigen-antibody interaction can not be predicted from the enthalpy contributed by van der Waals interac-

tions, hydrogen bonds or ionic bonds, alone. The entropy associated with the almost complete expulsion of water molecules from the interface represent an energy contribution similar in size. This means that perfect fit between the contours of the interacting molecules is a principal factor underlying antigen-antibody high affinity interactions.

Allergy vaccination

The concept of vaccination is based on two fundamental characteristics of the immune system, namely specificity and memory. Vaccination will prime the immune system of the recipient, and upon repeated exposure to the same proteins the immune system will be in a position to respond more rigorously to the challenge of for example a microbial infection. Vaccines are mixtures of proteins intended to be used in vaccination for the purpose of generating such a protective immune response in the recipient. The protection will comprise only components present in the vaccine and homologous antigens.

Compared to other types of vaccination allergy vaccination is complicated by the existence of an ongoing immune response in allergic patients. This immune response is characterized by the presence of allergen-specific IgE, the primary effect of which is the release of allergic symptoms upon exposure to allergens. Thus, allergy vaccination using allergens from natural sources has an inherent risk of side effects being in the utmost consequence life-threatening to the patient.

Approaches to circumvent this problem have been two-sided. One approach has led to the incorporation of the allergens into gel substances such as aluminium hydroxide. Aluminium hydroxide formulation has an adjuvant ef-

fect and a depot effect of slow allergen release reducing the tissue concentration of active allergen components. Another approach has been chemical modification of the allergens for the purpose of reducing allergenicity, i.e. IgE binding. Several approaches to chemical modification have been taken including 1. Chemical coupling of allergens to polymers, 2. Chemical cross-linking of allergens using formaldehyde, etc., 3. Allergen derived synthetic peptides. The rationale behind the two former approaches is to reduce IgE-binding while retaining immunogenicity, since successful allergy vaccination may partly rely on blocking IgG in addition to T cell effects. The rationale behind the use of peptides is also to change the response of allergen-specific T-cells, while reducing allergenicity. Other approaches based on this rationale has been the proposal of the use of "low IgE binding" isoforms. In recent years it has become clear that natural allergens are heterogeneous containing isoallergens and variants having up to approximately 25% of their amino acids substituted. Some isoallergens have been postulated to have reduced allergenicity and they have been proposed as candidates for safer allergy vaccination.

The mechanism behind successful allergy vaccination remains controversial. Most current approaches to artificial improvement in substances used for allergy vaccination focus on the allergen-specific T-cell as a target for therapy. T-cells play a key role in the overall regulation of immune responses. *In vitro* studies have indicated the possibility of altering the responses of allergen-specific T-cells by challenge with peptides containing relevant T-cell epitopes. The aim being to silence the T-cells (anergy induction) or to shift the response of the T-cells from the Th2-type leading to IgE production to the Th1-type leading to protective immune responses. However, a number of observations are commonly

reported from various clinical investigations of allergy vaccination : a) allergen-specific IgE remains the same during specific allergy vaccination (SAV), b) a new immune response (primarily IgG4) is initiated, but this response does not correlate with the onset of allergy symptom reductions, c) an allergen-specific Th0/1 T-cell response is initiated on top of the Th2 T-cell response, d) early phase skin prick reactions are transiently depressed whereas late phase skin prick reactions seem to be permanently suppressed. Given the above observations a new allergen-specific non-IgE antibody response is mounted upon SAV, this response matures during the SAV period until it reaches an average affinity which can compete efficiently with the IgE antibodies for the allergen binding. Since IgE antibodies reacts with native non-denatured allergens it follows that the 3D-structure of the allergens used for SAV essentially be retained in order to mount a non-IgE antibody response which can block the reaction between the allergen and IgE.

In vitro *mutagenesis and allergy vaccination*.

Attempts to reduce allergenicity by *in vitro* site directed mutagenesis have been performed using several allergens including Der f 2 (Takai *et al* 1997), Der p 2 (Smith *et al* 1997), a 39 kDa *Dermatophagoides farinae* allergen (Aki *et al* 1994), bee venom phospholipase A2 (Förster *et al* 1995), Ara h 1 (Burks *et al* 1997), Ara h 2 (Stanley *et al* 1997), *Bet v* 1 (Ferreira *et al* 1996 and 1998), birch profilin (Wiedemann *et al* 1996), and Ory s 1 (Alvarez *et al* 1995).

The rationale behind these approaches is identical to that outlined above. None of these studies have addressed the problem of conservation of the overall α-carbon backbone structure of the allergen upon mutagenesis.

OBJECT OF THE INVENTION

Rationale behind the present invention

The rationale behind the current invention takes a different starting point. According to this rationale the mechanism of successful allergy vaccination is not an alteration of the ongoing Th2-type immune response, but rather the initiation of a parallel and new Th1-type immune response. This model is supported by the observation that levels of specific IgE are unaffected by vaccination treatment. In addition, studies of nasal biopsies before and after allergen challenge do not show a reduction in T-cells with the Th2-like phenotype. Rather an increase in Th1-like T-cells are observed. Since the vaccine is administered sub-cutaneously and not through the airways, the new Th1-like immune response is thought to evolve in a location physically separated from the ongoing Th2 response thereby enabling the two responses to exist in parallel.

According to this rationale the vaccine must contain antibody-binding epitopes, which upon treatment will induce the production of protective IgG antibodies directed against epitopes which are cross-reactive with the natural allergens. For this purpose to be fulfilled it is essential that the allergen molecule retain its overall folding pattern after mutagenesis. The effects of vaccines are dose dependent. By reducing but not eliminating the IgE binding structures in the vaccine it may be possible to administrate higher doses in allergy vaccination leading to improved efficacy/safety ratios.

SUMMARY OF THE INVENTION

The present invention relates to the introduction of artificial amino acid substitutions into defined critical positions while retaining the overall folding pattern of the allergen.

The invention provides a recombinant allergen, which is a non-naturally occurring mutant derived from a naturally occurring allergen, wherein at least one surface-exposed, conserved amino acid residue of a B cell epitope is substituted by another residue which does not occur in the same position in the amino acid sequence of any known homologous protein within the taxonomic order from which said naturally occurring allergen originates, said mutant allergen having essentially the same three-dimensional structure as said naturally occurring allergen, and the specific IgE binding to the mutated allergen being reduced as compared to the binding to said naturally occurring allergen.

Such recombinant allergen is obtainable by

a) identifying amino acid residues in a naturally occurring allergen which are conserved with more than 70% identity in all known homologous proteins within the taxonomic order from which said naturally occurring allergen originates;

b) defining at least one patch of conserved amino acid residues being coherently connected over at least 400 $Å^2$ of the surface of the three-dimensional structure of the allergen molecule as defined by having a solvent accessibility of at least 20 %, said at least one patch comprising at least one B cell epitope; and

c) substituting at least one amino acid residue in said at least one patch by another amino acid being non-conservative in the particular position while essentially preserving the overall α-carbon backbone three-dimensional structure of the allergen molecule.

Preferably, specific IgE binding to the mutated allergen is reduced by at least 10% in average.

Recombinant allergens according to the invention may suitably be derived from inhalation allergens originating i.a. from trees, grasses, herbs, fungi, house dust mites, cockroaches and animal hair and dandruff. Important pollen allergens from trees, grasses and herbs are such originating from the taxonomic orders of *Fagales*, *Oleales* and *Pinales* including i.a. birch (*Betula*), alder (*Alnus*), hazel (*Corylus*), hornbeam (*Carpinus*) and olive (*Olea*), the order of *Poales* including i.a. grasses of the genera *Lolium*, *Phelum*, *Poa*, *Cynodon*, *Dactylis* and *Secale*, the orders of *Asterales* and *Urticales* including i.a. herbs of the genera *Ambrosia* and *Artemisia*. Important inhalation allergens from fungi are i.a. such originating from the genera *Alternaria* and *Cladosporium*. Other important inhalation allergens are those from house dust mites of the genus *Dermatophagoides*, those from cockroaches and those from mammals such as cat, dog and horse. Further, recombinant allergens according to the invention may be derived from venom allergens including such originating from stinging or biting insects such as those from the taxonomic order of Hymenoptera including bees, wasps, and ants.

Specific allergen components include e.g. *Bet v* 1 (*B. verrucosa*, birch), *Aln g* 1 (*Alnus glutinosa*, alder), *Cor a* 1 (*Corylus avelana*, hazel) and *Car b* 1 (*Carpinus betulus*, hornbeam) of the *Fagales* order. Others are *Cry j* 1

(*Pinales*), *Amb a* 1 and 2, , *Art v* 1 (*Asterales*), *Par j* 1 (*Urticales*), *Ole e* 1 (*Oleales*), *Ave e* 1, *Cyn d* 1, *Dac g* 1, *Fes p* 1, *Hol l* 1, *Lol p* 1, *Pas n* 1, *Phl p* 1 and 5, *Poa p* 1 and 2, *Sec c* 1 and *Sor h* 1 (various grass pollens), *Alt* a 1 and *Cla h* 1 (fungi), *Der f* 1 and 2, *Der p* 1 and 2 (house dust mites, *D. farinae* and *D. pteronyssinus*, respectively), *Bla g* 1 and 2, *Per a* 1 (cockroaches, *Blatella germanica* and *Periplaneta americana*, respectively), *Fel d* 1 (cat), *Can f* 1 (dog), *Equ c* 1, 2 and 3 (horse), *Apis m* 1 and 2 (honeybee), *Ves g* 1, 2 and 5, *Pol a* 1, 2 and 5 (all wasps) and *Sol i* 1, 2, 3 and 4 (fire ant).

The present invention also provides a method of preparing a recombinant allergen according to any one of claims 1-23, comprising

a) identifying amino acid residues in a naturally occurring allergen which are conserved with more than 70% identity in all known homologous proteins within the taxonomic order from which said naturally occurring allergen originates;

b) defining at least one patch of conserved amino acid residues being coherently connected over at least 400 $Å^2$ of the surface of the three-dimensional structure of the allergen molecule as defined by having a solvent accessibility of at least 20 %, said at least one patch comprising at least one B cell epitope, and

c) substituting at least one amino acid residue in said at least one patch by another amino acid being non-conservative in the particular position while essentially-preserving the overall α-carbon backbone three-dimensional structure of the allergen molecule.

In this method the best results are obtained by ranking the amino acid residues of said at least one patch with respect to solvent accessibility and substituting one or more amino acids among the more solvent accessible ones.

Generally, in the method according to the invention the substitution of one or more amino acid residues in said B cell epitope or said at least one patch is carried out by site-directed mutagenesis.

Further, the present invention provides a pharmaceutical composition comprising a recombinant allergen according to any one of claims 1-23 in combination with a pharmaceutically acceptable carrier and/or excipient, and optionally an adjuvant.

Such pharmaceutical composition may be in the form of a vaccine against allergic reactions elicited by a naturally occurring allergen in patients suffering from allergy.

DETAILED DESCRIPTION OF THE INVENTION

Critical criteria for substitution

The mutant carrying the substituted amino acid(s) should fulfil the following criteria:

1. The overall α-carbon backbone three-dimensional structure of the molecule should be conserved. Conserved is defined as an average root mean square deviation of the atomic coordinates comparing the structures below 2 %. This is important for two reasons: a) It is anticipated that the entire surface of the natural allergen constitutes an overlapping continuum of potential antibody-binding epitopes. The majority of the surface of the

molecule is not affected by the substitution(s), and thus retain its antibody-binding properties, which is important for the generation of new protective antibody specificities being directed at epitopes present also on the natural allergen. b) Stability, both concerning shelf-life and upon injection into body fluids.

2. The amino acid(s) to be substituted should be located at the surface, and thus be accessible for antibody-binding. Amino acids located on the surface are defined as amino acids in the three-dimensional structure having a solvent (water) accessibility of at least 20%. Solvent accessibility is defined as the area of the molecule accessible to a sphere with a radius comparable to a solvent (water, r = 1.4 Å) molecule.

3. The substituted amino acid(s) should be located in conserved patches larger than 400 $Å^2$. Conserved patches are defined as coherently connected areas of surface exposed conserved amino acid residues and backbone. Conserved amino acid residues are defined by sequence alignment of all known (deduced) amino acid sequences of homologues proteins within the taxonomical order. Amino acid positions having identical amino acid residues in more than 90% of the sequences are considered conserved. Conserved patches are expected to contain epitopes to which the IgE of the majority of patients is directed.

4. Within the conserved patches amino acids for mutagenesis should preferentially be selected among the most solvent (water) accessible ones located preferably near the centre of the conserved patch.

Preferentially, a polar amino acid residue is substituted by another polar residue, and a non-polar amino acid residue is substituted by another non-polar residue.

Preparation of vaccines is generally well-known in the art. Vaccines are typically prepared as injectables either as liquid solutions or suspensions. Such vaccine may also be emulsified or formulated so as to enable nasal administration. The immunogenic component may suitably be mixed with excipients which are pharmaceutically acceptable and further compatible with the active ingredient. Examples of suitable excipients are water, saline, dextrose, glycerol, ethanol and the like as well as combinations thereof. The vaccine may additionally contain other substances such as wetting agents, emulsifying agents, buffering agents or adjuvants enhancing the effectiveness of the vaccine.

Vaccines are most frequently administered parenterally by subcutaneous or intramuscular injection. Formulations which are suitable for administration by another route include oral formulations and suppositories. Vaccines for oral administration may suitably be formulated with excipients normally employed for such formulations, e.g. pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. The composition can be formulated as solutions, suspensions, emulsions, tablets, pills, capsules, sustained release formulations, aerosols, powders, or granulates.

The vaccines are administered in a way so as to be compatible with the dosage formulation and in such amount as will be therapeutically effective and immunogenic. The quantity of active component contained within the vaccine depends on the subject to be treated, i.a. the capability of the subjects immune system to respond to the treatment, the route of administration and the age and weight of the subject. Suitable dosage ranges can vary within the range from about 0.0001 µg to 1000 µg.

As mentioned above, an increased effect may be obtained by adding adjuvants to the formulation. Examples of such adjuvants are aluminum hydroxide and phosphate (alum) as a 0.05 to 0.1 percent solution in phosphate buffered saline, synthetic polymers of sugars used as 0.25 percent solution. Mixture with bacterial cells such as *C. parvum*, endotoxins or lipopolysaccharide components of gram-negative bacteria, emulsion in physiologically acceptable oil vehicles such as mannide monoaleate (Aracel A) or emulsion with 20 percent solution of a perfluorocarbon (e.g. Fluosol-DA) used as a block substitute may also be employed. Other adjuvants such as Freunds complete and incomplete adjuvants as well as QuilA and RIBI may also be used.

Most often, multiple administrations of the vaccine will be necessary to ensure an effect. Frequently, the vaccine is administered as an initial administration followed by subsequent inoculations or other administrations. The number of vaccinations will typically be in the range of from 1 to 50, usually not exceeding 35 vaccinations. Vaccination will normally be performed from biweekly to bimonthly for a period of 3 months to 5 years. This is contemplated to give desired level of prophylactic or therapeutic effect.

The recombinant allergen may be used as a pharmaceutical preparation, which is suitable for providing a certain protection against allergic responses during the period of the year where symptoms occur (prophylaxis). Usually, the treatment will have to be repeated every year to maintain the protective effect. Preparations formulated for nasal application are particular suited for this purpose.

EXAMPLE

Identification of common epitopes within Fagales pollen allergens

The major birch pollen allergen *Bet v* 1 shows about 90% amino acid sequence identity with major allergens from pollens of taxonomically related trees, i.e Fagales (for instance hazel and hornbeam) and birch pollen allergic patients often show clinical symptoms of allergic cross-reactivity towards these *Bet v* 1 homologous proteins.

Bet v 1 also shows about 50-60% sequence identity with allergic proteins present in certain fruits (for instance apple and cherry) and vegetables (for instance celery and carrot) and there are clinical evidence for allergic cross-reactivity between *Bet v* 1 and these food related proteins.

In addition, *Bet v* 1 shares significant sequence identity (20-40%) with a group of plant proteins called pathogenesis-related proteins (PR-10), however there are no reports of allergic cross-reactivity towards these PR-10 proteins.

Molecular modelling suggests that the structures of Fagales and food allergens and PR-10 proteins are close to be identical with the *Bet v* 1 structure.

The structural basis for allergic *Bet v* 1 cross-reactivity was reported in (Gajhede et al 1996) where three pathces on the molecular surface of *Bet v* 1 could be identified to be common for the known major tree pollen allergens. Thus, any IgE recognising these patches on *Bet v* 1 would be able to cross-react and bind to other Fagales major pollen allergens and give rise to allergic

symptoms. The identification of these common patches was performed after alignment of all known amino acid sequences of the major tree pollen allergens in combination with an analysis of the molecular surface of *Bet v* 1 revealed by the three-dimensional structure reported in (Gajhede et al 1996). In addition, the patches were defined to have a certain minimum size (>400 $Å^2$) based on the area covered by an antibody upon binding.

Selection of amino acid residues for site-directed mutagenesis.

Amino acid residues for site-directed mutagenesis were selected among residues present in *Bet v* 1 specific areas and the common patches since modifications of these is expected to affect the binding of serum IgE from the majority of patients showing clinical tree pollen allergic cross-reactivity.

The relative orientation and percentage of solvent-exposure of each amino acid residue within respective patch was calculated based on their atomic coordinates. Residues having a low degree of solvent exposure (<20%) were not regarded relevant for mutagenesis due to the possible disruption of the structure or lack of antibody interaction. The remaining residues were ranked according to their degree of solvent-exposure.

Sequence alignment

Sequences homologous to the query sequence (*Bet v* 1 No. 2801, WHO IUIS Nomenclature Subcommittee on Allergens) were derived from GenBank and EMBL sequence databases by a BLAST search (Altschul et al.). All sequences with BLAST reported probabilities less than 0.1 were taken into consideration and one list were constructed contain-

ing a non-redundant list of homologous sequences. These were aligned by CLUSTAL W (Higgins et al) and the percentage identity were calculated for each position in the sequence considering the complete list or taxonomically retaled species only. A total of 122 sequences were homologous to *Bet v 1 No. 2801* of which 57 sequences originates from taxonomically related species.

Cloning of the gene encoding Bet v 1.

RNA was was prepared from *Betula verrucosa* pollen (Allergon, Sweden) by phenol extraction and LiCl precipitation. Oligo(dT)-cellulose affinity chromatography was performed batchwise in Eppendorph tubes, and double-stranded cDNA was synthesized using a commercially available kit (Amersham). DNA encoding *Bet v* 1 was amplified by PCR and cloned. In brief, PCR was performed using cDNA as template, and primers designed to match the sequence of the cDNA in positions corresponding to the amino terminus of *Bet v* 1 and the 3'-untranslated region, respectively. The primers were extended in the 5'-ends to accommodate restriction sites (*Nco*I and *Hin*dIII) for directional cloning into pKK233-2.

Subcloning into pMAL-c.

The gene encoding *Bet v* 1 was subsequently subcloned into the maltose binding protein fusion vector pMAL-c (New England Biolabs). The gene was amplified by PCR and subcloned in frame with *malE* to generate maltose binding protein (MBP)-*Bet v* 1 protein fusion operons in which MBP and *Bet v* 1 were separated by a factor X_a protease cleavage site positioned to restore the authentic aminoterminal sequence of *Bet v* 1 upon cleavage, as described (Spangfort *et al* 1996a). In brief, PCR was performed using pKK233-3 with *Bet v 1* inserted as template and prim-

ers corresponding to the amino- and carboxyterminus of the protein, respectively. The promoter proximal primer was extended in the 5'-end to accommodate 4 codons encoding an in frame factor X_a protease cleavage site. Both primers were furthermore extended in the 5'-ends to accommodate restriction sites (*Kpn*I) for cloning. The *Bet v 1* encoding genes were subcloned using 20 cycles of PCR to reduce the frequency of PCR artefacts.

In vitro *mutagenesis*.

In vitro mutagenesis was performed by PCR using recombinant pMAL-c with *Bet v 1* inserted as template. Each mutant *Bet v 1* gene was generated by 3 PCR reactions using 4 primers.

Two mutation-specific oligonucleotide primers were synthesized accomodating each mutation, one for each DNA strand, see Figs. 1 and 2, Using the mutated nucleotide(s) as starting point both primers were extended 7 nucleotides in the 5'-end and 15 nucleotides in the 3'-end. The extending nucleotides were identical in sequence to the *Bet v 1* gene in the actual region.

Two generally applicable primers (denoted "all-sense" and "all non-sense" in Fig. 2) were furthermore synthesized and used for all mutants. These primers were 15 nucleotides in length and correspond in sequence to regions of the pMAL-c vector approximately 1 kilobase upstream and downstream from the *Bet v 1*. The sequence of the upstream primer is derived from the sense strand and the sequence of the downstream primer is derived from the non-sense strand, see Fig. 2.

Two independent PCR reactions were performed essentially according to standard procedures (Saiki *et al* 1988) with

the exception that only 20 temperature cycles were performed in order to reduce the frequency of PCR artefacts. Each PCR reaction used pMAL-c with *Bet v 1* inserted as template and one mutation-specific and one generally applicable primer in meaningful combinations.

The PCR products were purified by agarose gel electrophoresis and electro-elution followed by ethanol precipitation. A third PCR reaction was performed using the combined PCR products from the first two PCR reactions as template and both generally applicable primers. Again, 20 cycles of standard PCR were used. The PCR product was purified by agarose gel electrophoresis and electro-elution followed by ethanol precipitation, cut with restriction enzymes (*Bsi*WI/*Eco*RI), and ligated directionally into pMAL-c with *Bet v 1* inserted restricted with the same enzymes.

Figure 3 shows an overview of all *Bet v* 1 mutations.

Nucleotide sequencing.

Determination of the nucleotide sequence of the *Bet v* 1 encoding gene was performed before and after subcloning, and following *in vitro* mutagenesis, respectively.

Plasmid DNA's from 10 ml of bacterial culture grown to saturation overnight in LB medium supplemented with 0.1 g/l ampicillin were purified on Qiagen-tip 20 columns and sequenced using the Sequenase version 2.0 DNA sequencing kit (USB) following the recommendations of the suppliers.

Expression and purification of recombinant Bet v 1 and mutants.

Recombinant *Bet v* 1 (*Bet v* 1 No. 2801 and mutants) were over-expressed in *Escherichia coli* DH 5a fused to maltose-binding protein and purified as described in (Spangfort *et al* 1996a). Briefly, recombinant *E.coli* cells were grown at 37 °C to an optical density of 1.0 at 436 nm, whereupon expression of the *Bet v* 1 fusion protein was induced by addition of IPTG. Cells were harvested by centrifugation 3 hours post-induction, resuspended in lysis buffer and broken by sonication. After sonication and additional centrifugation, recombinant fusion protein was isolated by amylose affinity chromatography and subsequently cleaved by incubation with Factor Xa (Spangfort *et al* 1996a). After F Xa cleavage, recombinant *Bet v* 1 was isolated by gelfiltration and if found necessary, subjected to another round of amylose affinity chromatography in order to remove trace amounts of maltose-binding protein.

Purified recombinant *Bet v* 1 was concentrated by ultrafiltration to about 5 mg/ml and stored at 4 °C. The final yields of the purified recombinant *Bet v* 1 preparations were between 2-5 mg per liter *E. coli* cell culture.

The purified recombinant *Bet v* 1 preparations appeared as single bands after silver-stained SDS-polyacrylamide electrophoresis with an apparent molecular weight of 17.5 kDa. N-terminal sequencing showed the expected sequences as derived from the cDNA nucleotide sequences and quantitative amino acid analysis showed the expected amino acid compositions.

We have previously shown (Spangfort et al. 1996a) that recombinant *Bet v* 1 No. 2801 is immunochemically indistinguishable from naturally occurring *Bet v* 1.

Immunoelectrophoresis using rabbit polyclonal antibodies

The seven mutant *Bet v* 1 were produced as recombinant *Bet v* 1 proteins and purified as described above and tested for their reactivity towards polyclonal rabbit antibodies raised against *Bet v* 1 isolated from birch pollen. When analysed by immunoelectrophoresis (rocket-line immunoelectrophoresis) under native conditions, the rabbit antibodies were able to precipitate all mutants, indicating that the mutants had conserved three-dimensional structures. However, the presence of precipitate in the form of lines at the lower parts of the rockets of mutants Glu45Ser, Asn47Ser, Pro108Gly and Glu60Ser (see Fig. 3. DNA positions 133-134 GA-TC, 139-140 AA-TC, 321-323 CCC-TGG, 178-179 GA-TC, respectively), clearly indicates a partial identity (different epitope pattern) between the above mutants and recombinant *Bet v* 1.

These results suggested that non-naturally occurring substitutions introduced on the molecular surface of *Bet v* 1 can reduce a polyclonal antibody response raised against naturally occurring *Bet v* 1 without distortion of the overall three-dimensional allergen structure. In order to analyse the effect on human polyclonal IgE-response, the Glu45Ser mutant was selected for further analysis.

Bet v 1 Glu45Ser mutant

Glutamic acid in position 45 show a high degree of solvent-exposure (40%) and is located in a molecular surface patch common for Fagales allergens. A serine residue was found to occupy position 45 in some of the *Bet v* 1 ho-

mologous PR-10 proteins arguing for that glutamic acid can be replaced by serine without distortion of the three-dimensional structure. In addition, as none of the known Fagales allergen sequences have serine in position 45, the substitution of glutamic acid with serine gives rise to a non-naturally occurring *Bet v* 1 molecule.

T cell proliferation assay.

It was found that recombinant Glu45Ser *Bet v* 1 was able to induce proliferation in a T cell line from a birch pollen allergic patient. The analysis was carried out as described in Spangfort *et al* 1996a.

Crystallisation and structural determination of recombinant Glu45Ser Bet v 1.

Crystals of recombinant Glu45Ser *Bet v* 1 were grown by vapour diffusion at 25°C, essentially as described in (Spangfort *et al* 1996b). Glu45Ser *Bet v* 1, at a concentration of 5 mg/ml, was mixed with an equal volume of 2.0 M ammonium sulfate, 0.1 M sodium citrate, 1% (v/v) dioxane, pH 6.0 and equilibrated against 100x volume of 2.0 M ammonium sulfate, 0.1 M sodium citrate, 1% (v/v) dioxane, pH 6.0. After 24 hours of equilibration, crystal growth was induced by applying the seeding technique described in (Spangfort *et al* 1996b), using crystals of recombinant wild-type *Bet v* 1 as a source of seeds.

After about 2 months, crystals were harvested and analysed using X-rays generated from a Rigaku rotating anode as described (Spangfort *et al* 1996b) and the structure was solved using molecular replacement.

Structure of Bet v 1 Glu45Ser mutant.

The structural effect of the mutation was addressed by growing three-dimensional *Bet v* 1 Glu45Ser protein crystals diffracting to 3.0 Å resolution when analysed by X-rays generated from a rotating anode. The substitution of glutamic acid to serine in position 45 was verified by the *Bet v* 1 Glu45Ser structure electron density map which also showed that the overall structure is preserved.

IgE-binding properties of Bet v 1 Glu45Ser mutant.

The IgE-binding properties of *Bet v* 1 Glu45Ser mutant was compared with recombinant *Bet v* 1 in a fluid-phase IgE-inhibition assay using either a pool of serum IgE derived from birch allergic patients or individual serum collected from four allergic patients selected on the basis of a clinical history of allergy against both birch pollen and apple.

Recombinant *Bet v* 1 no. 2801 was biotinylated at a molar ratio of 1:5 (*Bet v* 1 no. 2801:biotin). The inhibition assay was performed as follows: a serum sample (25 µl) was incubated with solid phase anti IgE, washed, resuspended and further incubated with a mixture of biotinylated *Bet v* 1 no. 2801 (3.4 nM) and a given mutant (0-28.6 nM). The amount of biotinylated *Bet v* 1 no. 2801 bound to the solid phase was estimated from the measured RLU after incubation with acridiniumester labelled streptavidin. The degree of inhibition was calculated as the ratio between the RLU's obtained using buffer and mutant as inhibitor.

All sera were diluted to give a maximal response of 200 000 RLU when tested against a serum pool of birch allergic patients, all with very high specific IgE-content.

Figure 4 shows the inhibition of the binding of biotinylated recombinant *Bet v* 1 to serum IgE from a pool of allergic patients by non-biotinylated *Bet v* 1 and by *Bet v* 1 Glu45Ser mutant (Fig. 4a) and a representative inhibition pattern using sera from one out of the four birch pollen-apple allergic patients tested (Fig. 4b).

In both cases, the maximum level of inhibition reached by the mutant (about 75%) is lower compared to recombinant *Bet v* 1 (about 85%) indicating that some of the specific IgE present in the sera are unable to recognise mutant *Bet v* 1 Glu45Ser. The difference in maximum inhibition level was consistent for the serum pool even when the concentration of inhibiting proteins was increased up to 500 ng/50 µl (not shown).

There is also a clear difference in the amount of respective recombinant proteins necessary to reach 50% inhibition of the binding to serum IgE present both in the serum pool and in the sera from the individual patient. Recombinant *Bet v* 1 reaches 50% inhibition at a concentration of about 3 ng/50 µl whereas the corresponding concentration for *Bet v* 1 Glu45Ser is 5 ng/50 µl. This show that the single point mutation introduced in *Bet v* 1 Glu45Ser lowers the affinity of specific serum IgE by a factor of about 2.

REFERENCES

Aki T, Ono K, Hidaka Y, Shimonishi Y, Jyo T, Wada T, Yamashita M, Shigeta S, Murooka Y, Oka S: "Structure of IgE epitopes on a new 39-kD allergen molecule from the house dust mite, *Dermatophagoides farinae*". *Int Arch Allergy Immunol* **103**: 357-364 (1994).

Altschul SF, Gish W, Miller W, Myers EW, and Lipman DJ: "Basic local alignment search tool". J. Mol. Biol. **215**: 403-410 (1990).

Alvarez AM, Fukuhara E, Nakase M, Adachi T, Aoki N, Nakamura R, Matsuda T: "Four rice seed cDNA clones belonging to the alpha-amylase/trypsin inhibitor gene family encode potential rice allergens". *Biosci Biotechnol Biochem* **59**: 1304-1308 (1995).

Burks AW, Shin D, Cockrell G, Stanley JS, Helm RM, Bannon GA: "Mapping and mutational analysis of the IgE-binding epitopes on Ara h 1, a legume vicilin protein and a major allergen in peanut hypersensitivity". *Eur J Biochem* **245**: 334-339 (1997).

Ferreira F, Rohlfs A, Hoffmann-Sommergruber K, Schenk S, Ebner C, Briza P, Jilek A, Kraft D, Breitenbach M, Scheiner O: "Modulation of IgE-binding properties of tree pollen allergens by site-directed mutagenesis". *Adv Exp Med Biol* **409**: 127-135 (1996).

Ferreira F, Ebner C, Kramer B, Casari G, Briza P, Kungl AJ, Grimm R, Jahn-Schmid B, Breiteneder H, Kraft D, Breitenbach M, Rheinberger H-J, and Scheiner O: "Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy". *FASEB J.* **12**: 231-242 (1998).

Förster E, Dudler T, Gmachl M, Aberer W, Urbanek R, Suter M: "Natural and recombinant enzymatically active or inactive bee venom phospholipase A2 has the same potency to release histamine from basophils in patients with Hymenoptera allergy". *J Allergy Clin Immunol* 95: 1229-1235 (1995).

Gajhede M, Osmark P, Poulsen FM, Ipsen H, Larsen JN, Joost van Neerven RJ, Schou C, Løwnstein H, and Spangfort MD: "X-ray and NMR structure of *Bet v* 1, the origin of birch pollen allergy". nature structural biology 3: 1040-1045 (1996).

Higgins D, Thompson J, Gibson T, Thompson JD, Higgins DG, and Gibson TJ: "CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice". Nucleic Acids Res. 22: 4673-4680 (1994).

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA: "Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase". *Science* 239:487-491 (1988).

Smith AM, Chapman MD: "Localization of antigenic sites on Der p 2 using oligonucleotide-directed mutagenesis targeted to predicted surface residues". *Clin Exp Allergy* 27: 593-599 (1997).

Spangfort MD, Ipsen H, Sparholt SH, Aasmul-Olsen S, Larsen MR, Mørtz E, Roepstorff P, Larsen JN: "Characterization of Purified Recombinant *Bet v* 1 with Authentic N-terminus, Cloned in Fusion with Maltose-Binding Protein". *Prot Exp Purification* 8: 365-373 (1996a).

Spangfort MD, Larsen JN, Gajhede M: "Crystallization and Preliminary X-ray Investigation at 2.0 Å Resolution of *Bet v* 1, a Birch Pollen Protein Causing IgE-Mediated Allergy". *PROTEINS, Struc Func Genet* **26**: 358-360 (1996b).

Stanley JS, King N, Burks AW, Huang SK, Sampson H, Cockrell G, Helm RM, West CM, Bannon GA: "Identification and mutational analysis of the immunodominant IgE binding epitopes of the major peanut allergen Ara h 2". *Arch Biochem Biophys* **342**: 244-253 (1997).

Takai T, Yokota T, Yasue M, Nishiyama C, Yuuki T, Mori A, Okudaira H, Okumura Y: "Engineering of the major house dust mite allergen Der f 2 for allergen-specific immunotherapy". *Nat Biotechnol* **15**: 754-758 (1997).

Wiedemann P, Giehl K, Almo SC, Fedorov AA, Girvin M, Steinberger P, Rüdiger M, Ortner M, Sippl M, Dolecek C, Kraft D, Jockusch B, Valenta R: "Molecular and structural analysis of a continuous birch profilin epitope defined by a monoclonal antibody". *J Biol Chem* **271**: 29915-29921 (1996).

C L A I M S

1. Recombinant allergen, characterised in that it is a non-naturally occurring mutant derived from a naturally occurring allergen, wherein at least one surface-exposed, conserved amino acid residue of a B cell epitope is substituted by another residue which does not occur in the same position in the amino acid sequence of any known homologous protein within the taxonomic order from which said naturally occurring allergen originates, said mutant allergen having essentially the same three-dimensional structure as said naturally occurring allergen, and the specific IgE binding to the mutated allergen being reduced as compared to the binding to said naturally occurring allergen.

2. Recombinant allergen according to claim 1, characterised in that it is obtainable by

a) identifying amino acid residues in a naturally occurring allergen which are conserved with more than 70% identity in all known homologous proteins within the taxonomic order from which said naturally occurring allergen originates;

b) defining at least one patch of conserved amino acid residues being coherently connected over at least 400 $Å^2$ of the surface of the three-dimensional structure of the allergen molecule as defined by having a solvent accessibility of at least 20 %, said at least one patch comprising at least one B cell epitope; and

c) substituting at least one amino acid residue in said at least one patch by another amino acid being non-conservative in the particular position while essentially

preserving the overall α-carbon backbone three-dimensional structure of the allergen molecule.

3. Recombinant allergen according to claim 1 or 2, **characterised** in that the specific IgE binding to the mutated allergen is reduced by at least 10% in average.

4. Recombinant allergen according to any of claims 1-3, **characterised** in that when comparing the α-carbon backbone three-dimensional structures of the mutant and the naturally occurring allergen molecules, the average root mean square deviation of the atomic coordinates is below 2 %.

5. Recombinant allergen according to claim 2, **characterised** in that said at least one patch comprises atoms of 15-25 amino acid residues.

6. Recombinant allergen according to any one of claims 2-5, **characterised** in that the amino acid residues of said at least one patch are ranked with respect to solvent accessibility, and one or more amino acids among the more solvent accessible ones are substituted.

7. Recombinant allergen according to claim 6, **characterised** in that one or more amino acid residues of said at least one patch having a solvent accessibility of 30-80 % are substituted.

8. Recombinant allergen according to any one of claims 2-7, **characterised** in that 1-5 amino acid residues per 400 $Å^2$ in said at least one patch are substituted.

9. Recombinant allergen according to any one of claims 2-5, **characterised** in that the substitution of one or more amino acid residues in said B cell epitope or said

at least one patch is carried out by site-directed mutagenesis.

10. Recombinant allergen according to any one of claims 1-9, **characterised** in that it is derived from an inhalation allergen.

11. Recombinant allergen according to claim 10, **characterised** in that it is derived from a pollen allergen.

12. Recombinant allergen according to claim 10, **characterised** in that it is derived from a pollen allergen originating from the taxonomic order of *Fagales*, *Oleales* or *Pinales*.

13. Recombinant allergen according to claim 12, **characterised** in that it is derived from *Bet v* 1.

14. Recombinant allergen according to claim 13, **characterised** in that one amino acid residue of said B cell epitope or said at least one patch is substituted.

15. Recombinant allergen according to claim 14, **characterised** in that the substitution is Glu45Ser.

16. Recombinant allergen according to claim 11, **characterised** in that it is derived from a pollen allergen originating from the taxonomic order of *Poales*.

17. Recombinant allergen according to claim 11, **characterised** in that it is derived from a pollen allergen originating from the taxonomic order of *Asterales* or *Urticales*.

18. Recombinant allergen according to claim 10, characterised in that it is derived from a house dust mite allergen.

19. Recombinant allergen according to claim 18, characterised in that it is derived from a mite allergen originating from *Dermatophagoides*.

20. Recombinant allergen according to claim 10, characterised in that it is derived from a cockroach allergen.

21. Recombinant allergen according to claim 10, characterised in that it is derived from an animal allergen.

22. Recombinant allergen according to claim 21, characterised in that it is derived from an animal allergen originating from cat, dog or horse.

23. Recombinant allergen according to any one of claims 1-9, characterised in that it is derived from a venom allergen.

24. Recombinant allergen according to claim 23, characterised in that it is derived from a venom allergen originating from the taxonomic order of *Hymenoptera*.

25. A method of preparing a recombinant allergen according to any one of claims 1-24, characterised by

a) identifying amino acid residues in a naturally occurring allergen which are conserved with more than 70% identity in all known homologous proteins within the taxonomic order from which said naturally occurring allergen originates;

b) defining at least one patch of conserved amino acid residues being coherently connected over at least 400 $Å^2$ of the surface of the three-dimensional structure of the allergen molecule as defined by having a solvent accessibility of at least 20 %, said at least one patch comprising at least one B cell epitope; and

c) substituting at least one amino acid residue in said at least one patch by another amino acid being non-conservative in the particular position while essentially-preserving the overall α-carbon backbone three-dimensional structure of the allergen molecule.

26. A method according to claim 25, **characterised** by ranking the amino acid residues of said at least one patch with respect to solvent accessibility and substituting one or more amino acids among the more solvent accessible ones.

27. A method according to claim 25 or 26, **characterised** in that the substitution of one or more amino acid residues in said B cell epitope or said at least one patch is carried out by site-directed mutagenesis.

28. Recombinant allergen according to any of claims 1-24 for use as a pharmaceutical.

29. Pharmaceutical composition, **characterised** in that it comprises a recombinant allergen according to any one of claims 1-24, optionally in combination with a pharmaceutically acceptable carrier and/or excipient, and optionally an adjuvant.

30. A pharmaceutical composition according to claim 29, **characterised** in that it is in the form of a vaccine

against allergic reactions elicited by a naturally occurring allergen in patients suffering from allergy.

Fig. 1

Mutant-specific oligonucleotide primers used for mutant number 1. Mutated nucleotide underlined

```
                    10        20        30        40        50
                    |         |         |         |         |
Bet v 1 sense       5'- AATTATGAGACTGAGACCACCTCTGTTATCCCAGCAGCTCG -3'
Bet v 1 non-sense   3'- TTAATACTCTGACTCTGGTGGAGACAATAGGGTCGTCGAGC -5'
sense primer        5'-             TGAGACCCCCTCTGTTATCCCAG       -3'
non-sense primer    3'-    ATACTCTGACTCTGGGGGAGACA                -5'
```

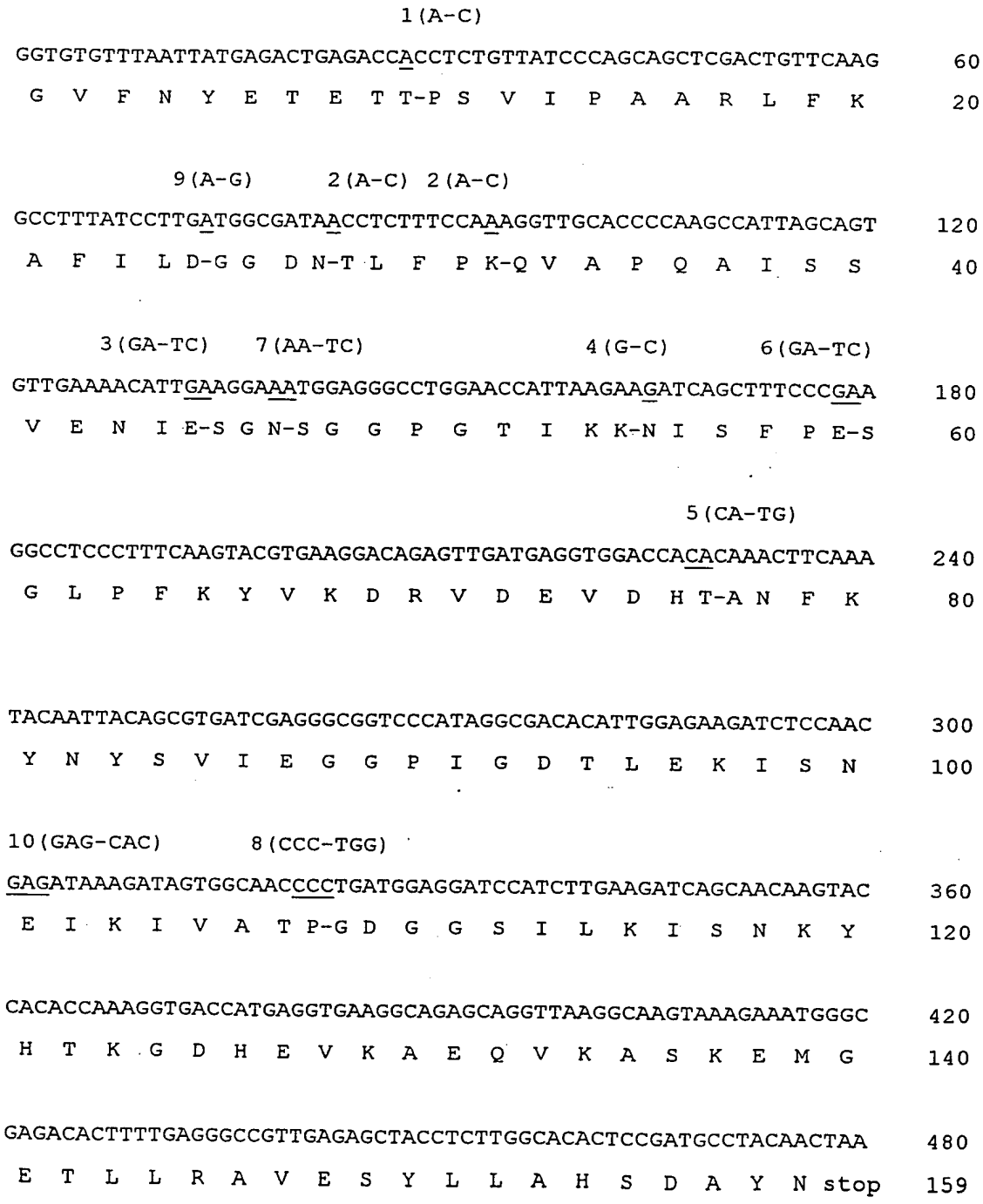
Fig. 2

Oligonucleotide primers for site directed mutagenesis of Bet v 1 (No. 2801).

all	sense	1: 183Bv, 15-mer	5'-GTTGCCAACGATCAG
1	sense	2: 184Bv, 23-mer	5'-TGAGACCCCCTCTGTTATCCCAG
1	non-sense	3: 185Bv, 23-mer	5'-ACAGAGGGGGTCTCAGTCTCATA
2	sense	4: 186Bv, 31-mer	5'-GATACCCTCTTTCCACAGGTTGCACCCCAAG
2	non-sense	5: 187Bv, 31-mer	5'-ACCTGTGGAAAGAGGGTATCGCCATCAAGGA
3	sense	6: 188Bv, 23-mer	5'-AACATTTCAGGAAATGGAGGGCC
3	non-sense	7: 189Bv, 23-mer	5'-TTTCCTGAAATGTTTTCAACACT
4	sense	8: 190Bv, 23-mer	5'-TTAAGAACATCAGCTTTCCCGAA
4	non-sense	9: 191Bv, 23-mer	5'-AGCTGATGTTCTTAATGGTTCCA
5	sense	10: 192Bv, 23-mer	5'-GGACCATGCAAACTTCAAATACA
5	non-sense	11: 193Bv, 23-mer	5'-AGTTTGCATGGTCCACCTCATCA
6	sense	12: 194Bv, 23-mer	5'-TTTCCCTCAGGCCTCCCTTTCAA
6	non-sense	13: 195Bv, 23-mer	5'-AGGCCTGAGGGAAAGCTGATCTT
7	sense	14: 196Bv, 24-mer	5'-TGAAGGATCTGGAGGGCCTGGAAC
7	non-sense	15: 197Bv, 24-mer	5'-CCCTCCAGATCCTTCAATGTTTTC
8	sense	16: 198Bv, 24-mer	5'-GGCAACTGGTGATGGAGGATCCAT
8	non-sense	17: 199Bv, 24-mer	5'-CCATCACCAGTTGCCACTATCTTT
all	non-sense	18: 200Bv, 15-mer	5'-CATGCCATCCGTAAG

Fig. 3

Overview of all mutations



Betv.fort.

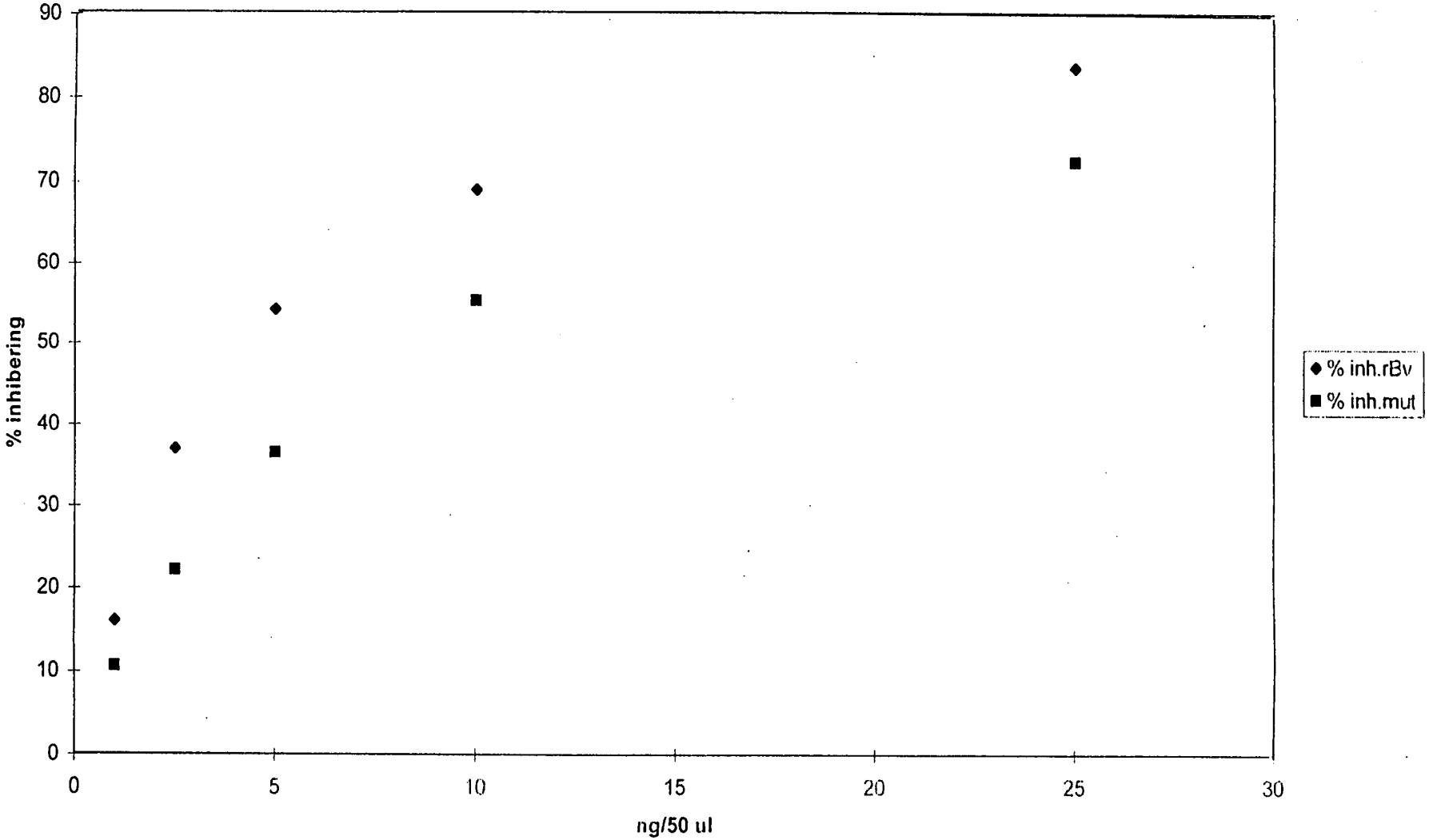


Fig. 4a

Pt.43_3

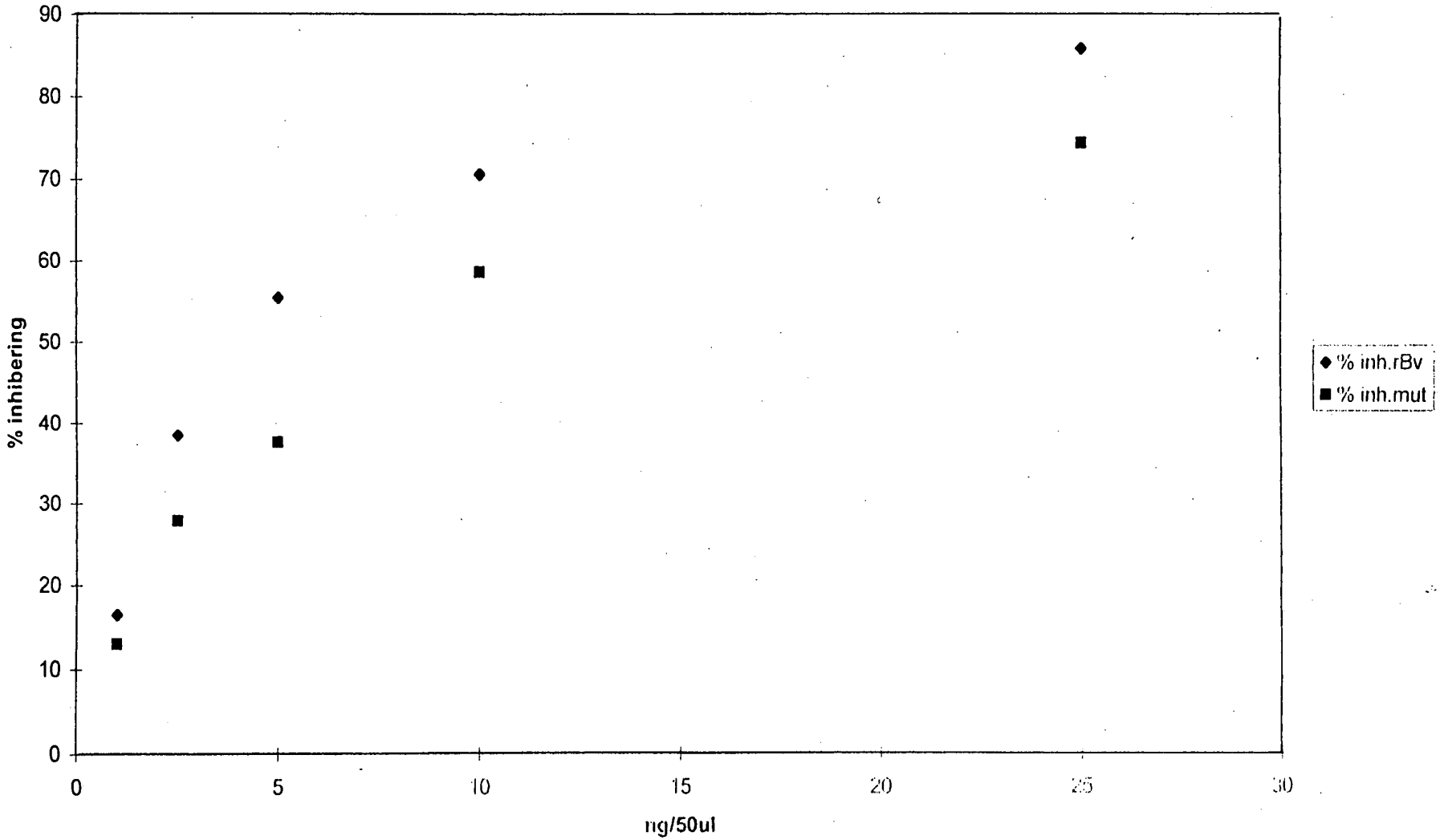

90
80
70
60
50
40
30
20
10
0
% inhibering
0
5
10
15
20
25
30
ng/50ul
% inh.rBv
% inh.mut

Fig. 4b